

FMRFamide-like Immunoreactivity in the Nervous System of the Starfish *Asterias rubens*

MAURICE R. ELPHICK*, ROLAND H. EMSON**, AND MICHAEL C. THORNDYKE*¹

**Wolfson Laboratory, Biology Department, Royal Holloway and Bedford New College, University of London, Egham, Surrey, TW20 OEX, and **Division of Biosphere Science, King's College, University of London*

Abstract. The nervous system of the starfish *Asterias rubens* was subjected to immunocytochemical investigation using antisera raised against the molluscan neuropeptide FMRFamide. Immunoreactivity was detected in the radial nerve cords and the circumoral nerve ring, as well as in the sub-epithelial nerve plexus of the tube foot system. The hyponeural part of the radial cords contained numerous immunoreactive cell bodies. In the ectoneural tissue, immunoreactive cells were present in the epithelium, with cell bodies especially abundant in the lateral parts of the nerve, close to the site of emergence of the innervation to the tube feet. The sub-epithelial nerve plexus of the tube feet contained immunoreactive fibers that were continuous with an extensive system of ectoneural immunoreactive fibers in the radial nerve cords. Immunoreactive fibers were particularly evident in the regenerating radial nerves of previously sectioned arms.

Introduction

The isolation and characterization of FMRFamide (Phe-Met-Arg-Phe-NH₂) from the clam *Macrocallista nimbosa* by Price and Greenberg (1977) heralded an era of intense investigation into both the occurrence and functional properties of this neuropeptide in molluscs. FMRFamide has a variety of effects on molluscan hearts, visceral and somatic muscles (reviewed by Greenberg *et al.*, 1983), and molluscan neurons (reviewed by Walker, 1986).

Since its discovery, antisera raised to FMRFamide have been used to investigate the possibility that FMRF-

amide-like peptides occur in non-molluscan species. Indeed, such immunochemical studies have revealed that FMRFamide-like substances are present in members of most of the major animal groups, including coelenterates (Grimmelikhuijzen, 1983), platyhelminthes (Reuter *et al.*, 1984; Gustafsson *et al.*, 1985), nemertines (Varndell and Polak, 1983), nematodes (Li and Chalfie, 1986; Cowden *et al.*, 1987), annelids (Kuhlman *et al.*, 1985; Porchet and Dhainaut-Courtois, 1988), crustaceans (Hooper and Marder, 1984; Jacobs and Van Herp, 1984), a chelicerate (Watson *et al.*, 1984), insects (Boer *et al.*, 1980), and vertebrates (Boer *et al.*, 1980; Dockray *et al.*, 1981). Subsequently, several of the peptides responsible for this immunoreactivity have been identified and, at present, the peptides isolated from protostomian species appear to be quite distinct from those of non-protostomes.

The Echinodermata, a major invertebrate phylum, has so far been neglected by those interested in neuropeptide biology. Therefore, as the first step in an attempt to identify FMRFamide-related peptides in echinoderms, we have carried out an immunocytochemical study of the distribution of FMRFamide-like material in the nervous system of the starfish, *Asterias rubens*.

Materials and Methods

Specimens of *Asterias rubens* were collected on the south coast of England, transported to Kings College, and maintained there in an aerated seawater system at 11°C.

The starfish were narcotized in 3.5% magnesium chloride, and the various parts of the nervous system were then dissected into cold (4°C) Bouin's fluid in seawater. After fixation for approximately 18 h at 4°C, the tissue

was embedded by routine methods in paraffin wax (58°C mp), sectioned at 7–15 μm , and mounted on poly-L-lysine coated glass slides. The primary rabbit antisera to FMRFamide (117I from C. J. P. Grimmelikhuijzen, and L135 from G. J. Dockray) were applied at dilutions between 1:100 and 1:1000. The 117I antiserum has been characterized by solid and liquid phase absorption tests with numerous, potentially cross-reactive peptides; it has high affinity for FMRFamide, as expected, but also some affinity for FLRFamide, FMKFamide, LTRPRYamide and RFamide (Grimmelikhuijzen, 1984).

Two methods were used to visualize the bound primary antibodies: application of a fluorescein isothiocyanate (FITC)-labelled swine anti-rabbit second antibody; or treatment with a peroxidase conjugated goat anti-rabbit second antibody, followed by rabbit peroxidase anti-peroxidase (PAP) complex and diaminobenzidine as the peroxidase substrate (the PAP method).

Three controls were carried out: the primary antibodies were pre-absorbed overnight at room temperature with 10 nmol of FMRFamide per ml of diluted antiserum; antibodies to other peptides (anti-insulin, anti-substance P, anti-cholecystinin) were tested; and non-immune serum was also examined.

Results

General morphology

The major components of the starfish nervous system are the circumoral nerve ring and its five branches—the radial nerve cords—which extend along the ventral surface of each arm (Fig. 1a). These nerve tracts contain two distinct parts, the ectoneural and hyponeural systems (Fig. 1b). The ectoneural system is further organized into an outer epithelial region containing cell bodies and supporting cells, and an inner axonal region traversed by fibers from the supporting cells (Fig. 1b). It is continuous with an extensive sub-epithelial nerve plexus of the skin, which is thickened locally to form the marginal nerve cords and the tube foot nerve ring (Fig. 1a). The hyponeural system lies above the ectoneural tissue, separated from it by a thin basement membrane (Fig. 1b).

A minor component of the starfish nervous system is the aboral nerve ring, which is continuous, in each arm, with the apical nerve, a small strand of tissue lying along the mid-dorsal region of the coelomic epithelium (Fig. 1a).

Immunocytochemistry

Positive FMRFamide-like immunoreactivity was detected in the circumoral nerve ring and radial nerve cords of *Asterias* (Fig. 2a, b).

Bipolar immunoreactive cell bodies were evident in

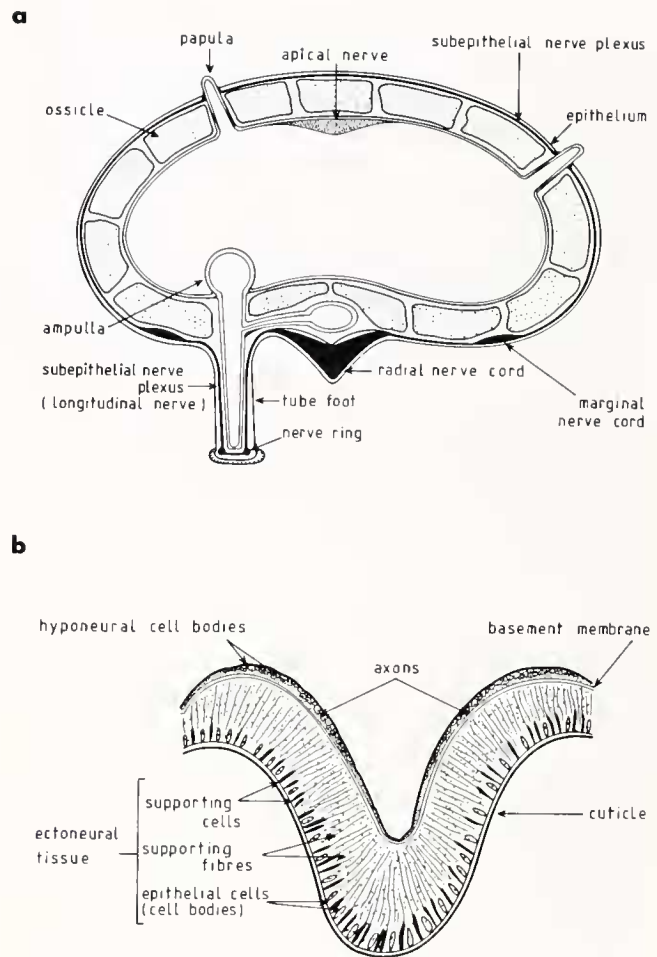


Figure 1. Diagrammatic representation of the nervous system in *Asterias*. (a) Composite cross section of an arm showing the anatomy of the nervous system at the level of a tube foot (left side) and between tube feet (right side). (b) Detailed cross section of radial nerve cord.

the ectoneural epithelium, interspersed between supporting cells (Fig. 2c). Longitudinal sections of the radial nerves show cell bodies along the entire length of the ectoneural network, with beaded fibers throughout the axonal region (Fig. 4a). Transverse sections reveal that the cell bodies and associated axonal tracts are particularly concentrated laterally, close to the points where the tube feet receive innervation from the nerve ring and cords (Figs. 2b, d; 3a).

Immunoreactive fibers occur throughout the sub-epithelial nerve plexus of the tube feet and are clearly continuous with the system of immunoreactive fibers in the ectoneural part of the adjacent nerve cord (Figs. 2b; 3a, b). No immunoreactive cell bodies were detected in the nerve plexus of the tube feet.

In the hyponeural part of the nervous system, immunoreactive cell bodies are particularly abundant, and processes could occasionally be seen directed towards the

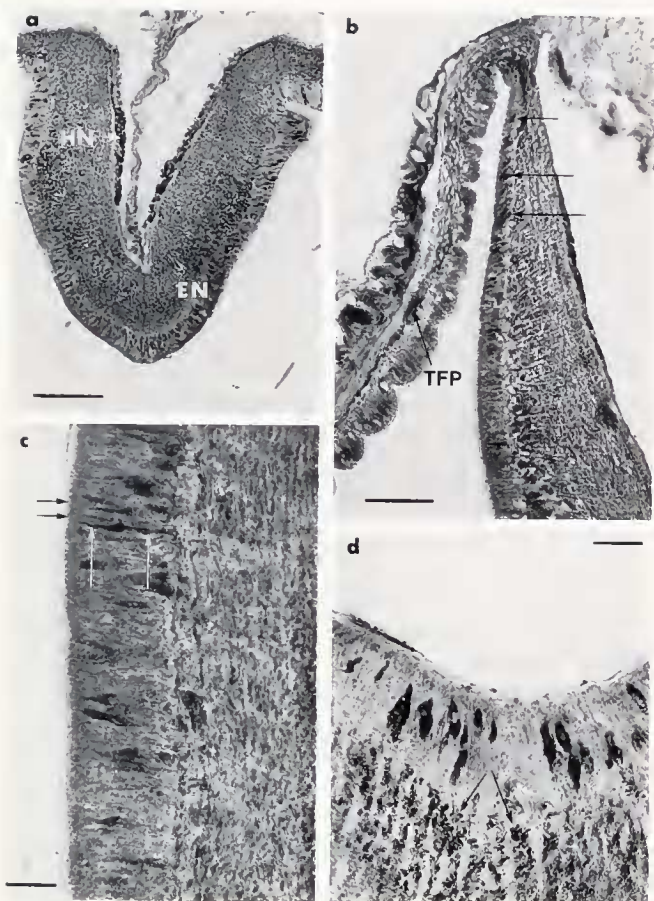


Figure 2. (a) Transverse section of radial nerve showing distribution of immunoreactivity in the ectoneural (EN) and hyponeural (HN) systems. Primary antibody, 1171, with PAP labelling. Scale bar = 100 μm .

(b) Longitudinal section of circumoral ring and part of tube foot epithelium showing lateral concentration of ectoneural cell bodies (triple arrows) and distinctive tube foot sub-epithelial nerve plexus (TFP). Primary antibody, 1171, with PAP labelling. Scale bar = 100 μm .

(c) Longitudinal section of radial nerve cord showing bipolar ectoneural cell bodies (white arrows) interspersed between supporting cells (black arrows) of the epithelium. Primary antibody, 1171, with PAP labelling. Scale bar = 30 μm .

(d) Transverse section of lateral region of the radial nerve cord near junction with tube foot showing increased concentration of immunoreactive ectoneural cell bodies and fibers (arrows). Primary antibody, L135, with PAP labelling. Scale bar = 30 μm .

basement membrane, although no fibers appeared to cross it in either direction (Fig. 4a, c).

In preliminary experiments designed to investigate the pattern of neuronal regeneration in previously sectioned arms, the concentration of immunoreactive fibers in the ectoneural system of the regenerates was noticeably increased (Fig. 4b).

Both FMRFamide antisera used gave positive results, but all of the control experiments, including those using

antisera previously absorbed with FMRFamide, proved negative.

Discussion

This investigation records for the first time, the occurrence of immunoreactive FMRFamide-like molecules in the nervous system of an echinoderm. These findings have implications for both neuropeptide phylogeny and echinoderm neurobiology.

Neuropeptide phylogeny

Over the last decade, FMRFamide-like peptides have been characterized in a variety of species and appear, at present, to fall into two distinct groups. First, those isolated from protostome phyla (Nematoda, Annelida, Mollusca, and Arthropoda) share with FMRFamide the general C-terminal sequence: F(X)RFamide, where X can be methionine, leucine, or isoleucine. Second, those peptides isolated from non-protostomes (Coelenterata and Chordata) usually share with FMRFamide only the C-terminal RFamide.

Greenberg *et al.* (1988) suggested that the protostomian peptides are homologous, whereas the sharing of the RFamide C-terminus with FMRFamide among the non-protostomian peptides "may merely reflect general characteristics of associations between peptides and proteins. If there is a homology, it is likely to reside with the class of membrane proteins comprising peptide receptors." Since the echinoderms are deuterostomian inver-

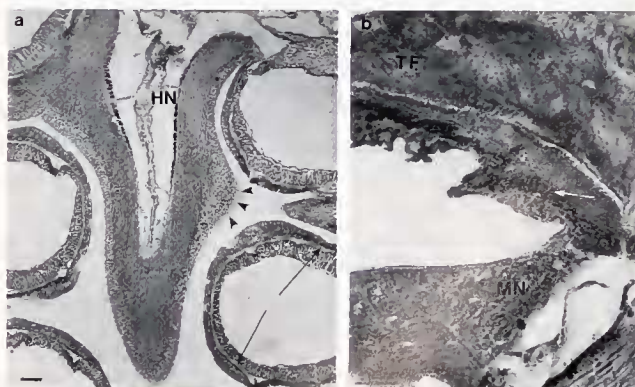


Figure 3. (a) Oblique section through radial nerve cord. Hyponeural cell bodies (HN) are clearly evident as is the sub-epithelial plexus in adjacent tube feet (arrows). Notice the high concentration of immunoreactive cells where the nerve cord branches to innervate the tube feet (arrow heads). Primary antiserum, 1171, with PAP labelling. Scale bar = 100 μm .

(b) Transverse section of marginal nerve cord (MN) and adjacent tube foot (TF). Immunoreactive fibers in the sub-epithelial plexus are clearly evident (arrow). Primary antiserum, 1171, with PAP labelling. Scale bar = 30 μm .

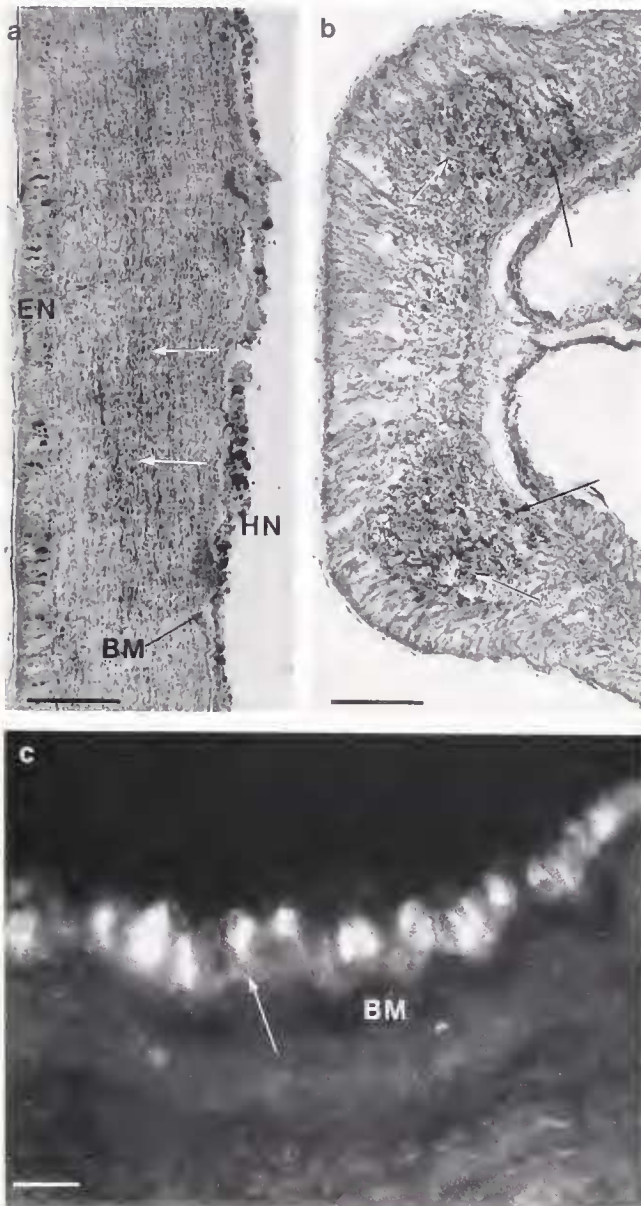


Figure 4. (a) Longitudinal section of radial nerve with cell bodies in both ectoneural (EN) and hyponeural (HN) systems as well as beaded fibers in the axonal region (arrows). BM, basement membrane. Primary antibody, L135, with PAP labelling. Scale bar = 100 μ m.

(b) Transverse section of regenerating radial nerve to show dense concentration of immunoreactive fibers (arrows). The absence of immunoreactive cell bodies suggests these fibers are derived from cells distal to the region of growth. Primary antibody, L135, with PAP labelling. Scale bar = 100 μ m.

(c) Longitudinal section of the radial nerve cord showing hyponeural cell bodies, one with a process (arrow) directed toward the basement membrane (BM). Primary antibody, 1171, with FITC labelling. Scale bar = 25 μ m.

tebrates, the characterization of the FMRFamide-like peptides in echinoderms would provide a further test of the notion that the F(X)RFamide peptides are peculiar to protostomes.

Echinoderm neurobiology

Our current understanding of echinoderm neurobiology is far behind that of most of the other major invertebrate phyla (see Cobb, 1987, 1988). Only one native echinoderm neuropeptide has ever been thoroughly investigated: gonad stimulating substance (GSS) (see Kanatani, 1979). Discovered thirty years ago (Chaet and McConnaughey, 1959), GSS has only recently (and partially) been sequenced (Shirai, 1987).

This is the first extensive study of peptidergic neurons in an echinoderm. The distribution of FMRFamide-like immunoreactivity in the nervous system may provide some clues as to the function of these peptides in starfish. The abundance of immunoreactivity and its presence in both the ectoneural and hyponeural systems suggests that the peptides may have a general transmitter-like role. However, the immunoreactivity is particularly associated with the innervation of the tube feet. Thus the sub-epithelial nerve plexus of the tube feet contains numerous immunoreactive fibers, whereas the soma of these neurons appear to lie within the adjacent nerve cord or ring.

Florey and Cahill (1980) demonstrated that the tube feet of sea urchins are under cholinergic motor control. Their evidence indicates that chemical transmission involves the diffusion of acetylcholine (ACh) from nerve terminals of the sub-epithelial plexus to the musculature, across the intervening connective tissue layer. Peptides produced by the immunoreactive neurons described here may be modulating the motor control of tube foot activity. Some circumstantial support for this idea comes from Unger's work (1960, 1962). Using simple chromatographic methods, this author isolated two physiologically active substances from the radial nerve cords of *Asterias glacialis*. In addition to effecting color changes, both factors induced movement in whole animals, as well as in isolated arms, and one of them strongly excited the *Helix* heart. These effects were clearly distinguishable from those of ACh, serotonin, adrenalin, noradrenalin, and histamine, and we speculate that the extracts may have contained peptidic factors, including FMRFamide-like molecules.

The immunocytochemical data presented in this report shows us relatively little about the chemical nature of the immunoreactive peptides since any peptide with a C-terminal sequence similar to that of FMRFamide might cross-react with the antisera. A good example is the family of pancreatic polypeptide-related peptides (PP-RP) which terminates in Arg-Tyr-amide (see Thorn dyke, 1986, for a more complete discussion of this problem).

Experiments designed to isolate and sequence the FMRFamide-like peptides in *Asterias* are currently un-

derway. Once one or more sequences are known and synthesized, the physiological roles of the native echinoderm molecules can be established.

Note added in proof

Three novel peptides, detected with antisera to FRMFamide, have now been purified from the radial nerves of the starfish *Asterias*, and sequenced (Elphick *et al.*, 1989).

Acknowledgments

We thank Professor M. J. Greenberg, for reading the manuscript and offering helpful criticism, and Professor G. J. Dockray and Dr. C. J. P. Grimmelikhuijzen, for gifts of antisera. Thanks also to Pat Enser and Zyg Podhorodecki for help with manuscript preparation. This work was partly supported by grants from the Science and Engineering Research Council, Nuffield Foundation, and Royal Society (to MCT).

Literature Cited

- Boer, H. H., L. P. C. Schot, J. A. Veenstra, and D. Reichelt. 1980. Immunocytochemical identification of neural elements in the central nervous system of a snail, some insects, a fish and a mammal with an antiserum to the molluscan cardio-excitatory tetrapeptide FMRF-amide. *Cell Tissue Res.* **213**: 21–27.
- Chaet, A. B., and R. A. McConnaughey. 1959. Physiologic activity of nerve extracts. *Biol. Bull.* **117**: 407.
- Cobb, J. L. S. 1987. Neurobiology of Echinodermata. Pp 483–525 in *Nervous Systems in Invertebrates*, M. A. Ali, ed. Plenum Press, New York and London.
- Cobb, J. L. S. 1988. Neurohumors and neurosecretion in echinoderms: a review. *Comp. Biochem. Physiol.* **91C**: 151–158.
- Cowden, C., P. Sithinrgul, J. Guastella, and A. Stretton. 1987. FMRFamide-like peptides in *Ascaris suum*. *Am. Zool.* **27**: 127A.
- Dockray, G. J., C. Vaillant, R. G. Williams, R. J. Gayton, and N. Osborne. 1981. Vertebrate brain-gut peptides related to FMRFamide and Met-enkephalin-Arg⁷Phe¹. *Peptides* **2**(suppl.2): 25–30.
- Elphick, M. R., D. A. Price, T. D. Lee, and M. C. Thorndyke. 1989. The SALMFamides: a new family of neuropeptides isolated from an echinoderm. *Soc. Neurosci. Abstr.* **15**: (in press).
- Florey, E., and M. A. Cahill. 1980. Cholinergic control of sea urchin tube feet: evidence for chemical transmission without synapses. *J. Exp. Biol.* **88**: 281–292.
- Greenberg, M. J., S. D. Painter, K. E. Doble, G. T. Nagle, D. A. Price, and H. K. Lehman. 1983. The molluscan neurosecretory peptide FMRFamide: comparative pharmacology and relationship to the enkephalins. *Fed. Proc.* **42**: 82–86.
- Greenberg, M. J., K. Payza, R. J. Nachman, G. M. Holman, and D. A. Price. 1988. Relationships between the FMRFamide-related peptides and other peptide families. *Peptides* **9**(Suppl.1): 125–135.
- Grimmelikhuijzen, C. J. P. 1983. FMRFamide immunoreactivity is generally occurring in the nervous systems of coelenterates. *Histochemistry* **78**: 361–381.
- Grimmelikhuijzen, C. J. P. 1984. Peptides in the nervous system of coelenterates. Pp 39–58 in *Evolution and Tumour Pathology of Neuroendocrine System*, S. Falkmer, R. Håkanson, and F. Sundler, eds. Elsevier, Amsterdam.
- Gustafsson, M. K. S., M. C. Wikgren, T. J. Karhi, and L. P. C. Schot. 1985. Immunocytochemical demonstration of neuropeptides and serotonin in the tapeworm *Diphyllobothrium dendriticum*. *Cell Tissue Res.* **241**: 171–182.
- Hooper, S. L., and E. Marder. 1984. Modulation of a central pattern generator by two neuropeptides, proctolin and FMRFamide. *Brain Res.* **305**: 186–191.
- Jacobs, A. C., and F. Van Herp. 1984. Immunocytochemical localization of a substance in the eyestalk of the prawn, *Palaemon serratus*, reactive with an anti-FMRFamide rabbit serum. *Cell Tissue Res.* **235**: 601–605.
- Kanatani, H. 1979. Hormones in echinoderms. Pp. 273–307 in *Hormones and Evolution*, E. J. W. Barrington, ed. Academic Press, London.
- Kuhlman, J. R., C. Li, and R. L. Calabrese. 1985. FMRFamide-like substances in the leech: 1. Immunocytochemical localization. *J. Neurosci.* **5**: 2301–2309.
- Li, C., and M. Chalfie. 1986. FMRFamide-like immunoreactivity in *C. elegans*. *Soc. Neurosci. Abstr.* **12**: 246.
- Porchet M., and N. Dhainaut-Courtois. 1988. Neuropeptides and monoamines in annelids. Pp 219–234 in *Neurohormones in Invertebrates*, M. C. Thorndyke and G. J. Goldsworthy, eds. Cambridge University Press, Cambridge, U.K.
- Price, D. A., and M. J. Greenberg. 1977. Structure of a molluscan cardioexcitatory neuropeptide. *Science* **197**: 670–671.
- Reuter, M., T. Karhi, and L. P. C. Schot. 1984. Immunocytochemical demonstration of peptidergic neurons in the central and peripheral nervous systems of the flatworm *Microstomum lineare* with anti-serum to FMRFamide. *Cell Tissue Res.* **238**: 431–436.
- Shirai, H., P. Bulet, N. Kondo, M. Isobe, K. Imai, T. Goto, and I. Kubota. 1987. Gonad-stimulating substance of starfish. *Gen. Comp. Endocrinol.* **66**: 50.
- Thorndyke, M. C. 1986. Immunocytochemistry and evolutionary studies with particular reference to peptides. Pp. 308–327 in *Immunocytochemistry: Modern Methods and Applications*, J. M. Polak and S. Van Noorden, eds. J. Wright and Sons, Bristol.
- Unger, H. 1960. Neurohormone bei seesternen *Marthasterias glacialis*. *Symp. Biol. Hung.* **1**: 203–207.
- Unger, H. 1962. Experimentelle und histologische Untersuchungen über Wirkfaktoren aus dem Nervensystem von *Asterias (Marthasterias) glacialis*. (Asteroidea, Echinodermata). *Zool. Jahrb. Abt. Allg. Zool. Physiol. Tiere* **3** **69**: 481–536.
- Varndell, I. M., and J. M. Polak. 1983. Glycolytic enzymes and a peptide closely related to FMRFamide are present in neurosecretory cells in nemertean cerebral ganglia. *Am. Zool.* **23**: 924.
- Walker, R. J. 1986. Transmitters and modulators. Pp 279–485 in *The Mollusca, Vol. 9, Part 2*, A.O.D. Willows, ed. Academic Press, New York.
- Watson, W. H., J. R. Groome, B. M. Chronwall, J. Bishop, and T. L. O'Donohue. 1984. Presence and distribution of immunoreactive and bioactive FMRFamide-like peptides in the nervous system of the horseshoe crab, *Limulus polyphemus*. *Peptides* **5**: 585–595.